Quality evaluation of Propolis. 1. A comparative study on radical scavenging effects of Propolis and Vespae Nidus (露蜂房)

Katsumichi MATSUSHIGE, Ines Tomoco KUSUMOTO, Yuriko YAMAMOTO, Shigetoshi KADOTA* and Tsuneo NAMBA

Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University

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Abstract

Propolis is a resinous material collected by the honey bee that has been used as a valuable folk medicine due to its medicinal properties such as antiinflammatory, antimicrobial and immunostimulatory properties. On the other hand, Vespae Nidus (露蜂房) is a crude drug used in Chinese traditional medicine that has some properties similar to those of propolis. The pharmacological effects of propolis are thought to be related at least in part, with the free radical scavenging effects. By testing the propolis collected in different regions of Brazil and Vespae Nidus for DPPH radical and superoxide anion scavenging effects both were found to possess such effects, and the most potent scavenging action was shown by the EtOH-insoluble fraction of their water extracts.

Key words Propolis, Vespae Nidus, radical scavenging.

Abbreviations BSA, bovine serum albumin ; DPPH, 1,1-diphenyl-2-picrylhydrazyl ; IC_{50}, 50 % inhibitory concentration ; NBT, nitroblue tetrazolium ; PMS, phenazyne methosulfate ; SOD, superoxide dismutase ; XOD, xanthine oxidase.

Introduction

Since ancient times the human being has received many benefits from the honey bees, whose valuable products have been used as nourishment and medicines. Propolis is a resinous material that the bee collects from the exudates of plants and attaches to the beehive and has been an old folk medicine. It is reported to have some medicinal properties as antimicrobial, antiinflammatory and immunostimulatory properties.[1] In the last few years propolis has been gaining popularity in Japan although its use is restricted as a dietary natural product. However, it faces a serious problem on the quality evaluation since propolis is subject to great variations according to the species of the honey bee and the variety of the plants surrounding the apiaries.

The efficacy of propolis as folk medicine reported so far led us to study an old Chinese traditional medicine derived from the hornet nest, the Vespae Nidus (露蜂房). A brief historical review of both natural medicines showed that they have some features in common such as the similar source in nature and medicinal properties. Vespae Nidus is known to possess antimicrobial, antiinflammatory, and tumor healing properties.[2]

Recently propolis from Poland[3] and Cuba[4] were reported to present free radical scavenging effects and these findings were applied to compare the biological activity of propolis from Brazil and the Chinese crude drug Vespae Nidus. Moreover, in order to find out parameters for standardization of propolis, Brazilian propolis of different regions were compared in some tests for the radical scavenging effects.

*〒930-01 富山市杉谷2630
富山医科薬科大学和漢薬研究所 資源開発部門 門田重利
2630 Sugitani, Toyama 930-01, Japan

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Materials and Methods

Propolis and Vespae Nidus : The sources of Brazilian propolis and Vespae Nidus are shown in Table I. Propolis collected at different areas in Brazil were provided by Nihon Propolis Co., Ltd. (Tokyo). Vespae Nidus from the market of Hong Kong was purchased from Uchida Pharmaceutical Co., Ltd. (Tokyo). This crude drug was identified to be the nest of *Vespula flaviceps* SMITH (Family Vespidae) by Dr. Masami Sasaki, Institute of Honeybee Science, Faculty of Agriculture of Tamagawa University (Japan).

Preparation of the extracts : The extracts and fractions of propolis were prepared as illustrated in Chart 1. The same method was applied to Vespae Nidus. A selection of this crude drug was made prior to the extraction by removing the impurities and organic residues of hornet. Ten grams of each sample were extracted two times with 100 ml of hot water (80°C/3 h), filtered, concentrated under reduced pressure and freeze dried. The residue of the water extract was extracted two times with 100 ml of MeOH (reflux/3 h) and the solvent was evaporated under reduced pressure. The water extract was mixed with 10 volumes of EtOH and centrifuged to separate into EtOH-soluble and -insoluble fractions. The water extract of Vespae Nidus gave only traces of EtOH-soluble fraction, which was excluded from the assays. Similarly the MeOH extract was separated into ether-soluble and -insoluble fractions. A voucher specimen of each sample has been deposited in the Museum of Materia Medica of Toyama Medical and Pharmaceutical University. Each extract and fraction was dissolved in water or EtOH before adding to the reaction mixture.

Enzymes : Superoxide dismutase (SOD, Cu, Zn type) and xanthine oxidase (XOD, from butter milk) were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan).

Chemicals : Xanthine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT), EDTA 2 Na, phenazyne methosulfate (PMS) and allopurinol were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). Caffeic acid was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo), bovine serum albumin (BSA), from Seikagaku Corporation

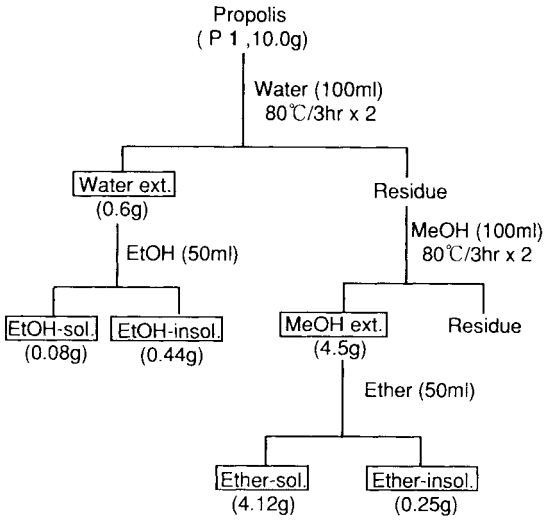

Chart 1 Extraction and fractionation of propolis.

Table I Samples of Propolis and Vespae Nidus and their geographical source.

Sample	Producing Area	Main Vegetation
Propolis	(Brazil)	
P1	Lagoa Vermelha, RS	*Araucaria* spp., primeval forest
P2	Atibaia, SP	*Eucaliptus* spp., forest
P3	Corumbatai, SP	*Citrus* spp., *Eucaliptus spp.*, primeval forest
P4	Carlos Barbosa, RS	Natural Eucaliptus forest, *Araucaria* spp.
P5	Carlos Barbosa, RS	Several varieties of *Eucaliptus* spp.
Vespae Nidus (露蜂房)		
R1	Market of Hong Kong	

RS = Rio Grande do Sul State ; SP = São Paulo State

(Tokyo), and NADH disodium salt from Sigma Chemical Co. (St. Louis, MO, USA).

Analytical apparatus and chromatography : UV spectrometer model UV-2200 Shimadzu (Shimadzu Corporation, Kyoto, Japan). A two-dimensional thin-layer chromatography (TLC) was performed in Avicel SF plates (Funakoshi, Tokyo), developing with BuOH : HOAc : H_2O (4 : 1 : 5) in the first direction and with HOAc : H_2O (15 : 85) in the second direction. The spray reagents used were 5 % $FeCl_3$ or 2 % ninhydrin.

Photometric determination of free radicals : The aqueous extracts and fractions were dissolved in water and the MeOH extracts and EtOH or ether-soluble fractions were dissolved in EtOH. The final concentration of EtOH in the reaction mixture was 1 % and this solvent had no influence on the absorbance in the test for DPPH radical or in the test for superoxide anion in a concentration up to 2.5 %. In each experiment indicated below the absorbance was measured against a blank without the test sample and the control reaction contained the same solvent used to dilute the test sample. The absorbance of the test sample was given by the difference between the absorbance of the reactions with and without test sample.

1. *DPPH radical* : The scavenging effect corresponded to the intensity of quenching DPPH, as described by Hatano *et al.*.[5] A portion of the sample solution was mixed with the same volume of 6×10^{-5}M DPPH in EtOH and allowed to stand for 30 min at room temperature. The absorbance was then measured at 520 nm. Caffeic acid was used as a positive control and the IC_{50} obtained in this condition was 1.0 μg/ml.

2. *Superoxide anion* : The SOD-like activity of the samples were measured by two different methods :

i) *Reaction of xanthine and XOD* : The production of superoxide anions in xanthine-XOD system was calculated following the methods of Imanari *et al.*[6] A reaction mixture composed of 0.1 ml of each 0.05M Na_2CO_3 (pH 10.2), 3 mM xanthine, 3 mM EDTA, 1.5 mg/ml BSA, 0.75 mM NBT, the test sample, and 0.1 ml of 0.14 mg/ml XOD was incubated 20 min at 25 °C. Then the reaction was stopped with 0.1 ml of 6 mM $CuCl_2$ and the absorbance was measured at 560 nm. At this condition SOD inhibited the production of superoxide anion by 50 % at a concentration of 8.0×10^{-3} μg/ml. Caffeic acid was used as a positive control (IC_{50} = 1.2 μg/ml).

ii) *Non-enzymatic reaction* : The method required NADH and PMS for production of superoxide anion, according to Nishikimi *et al.*[7] In a 1.0 ml reaction mixture composed of 100 mM phosphate buffer (pH 7.4), 0.01 mM of PMS, 0.025 mM NBT and the test sample, 0.1 mM of 1.56 mM NADH was mixed and the absorbance at 560 nm was measured after 2 min. The concentration of SOD required to inhibit the production of the superoxide anion by 50 % in this reaction was 2.6 μg/ml. Caffeic acid was used as a positive control (IC_{50} = 36 μg/ml).

Determination of XOD activity : Following the method of Robak and Gryglewski,[8] the test sample was added to a reaction mixture of 50 mM phosphate buffer (pH 7.8), 0.1 mM xanthine, and 0.04 units/ml (0.1 mg/ml) XOD. The production of uric acid in this reaction was measured by the absorbance at 295 nm. Allopurinol was used as a positive control (IC_{50} = 4.0 μg/ml).

Radical scavenging effects : The percent scavenging effect was determined as the ratio of the difference of absorbance between the test solution and its blank to the control solution. The result is the mean of 4 measurements. Dunnett's test was used for the statistical analysis of the data and the test sample values were considered to be significantly different from the corresponding control values at the statistical significance of $p<0.05$.

Results

Preliminary chemical analysis

The percent (w/w) yield of the extracts and fractions of propolis (**P1** **P5**) and Veapae Nidus (**R1**) is shown in Table II. There was a distinct difference on the yield of the water and the MeOH extracts of propolis. While 6.0 - 7.48 % of water extract were obtained, the yield of MeOH extract was 35.0-43.7 %. There was no remarkable differences on the yield between propolis of different sources. On the contrary, the yield of **R1** extracts was higher in water extraction (24.16 %) than that in MeOH extraction (4.46%). The samples of propolis (**P1-P5**) and Vespae Nidus (**R1**) were studied qualitatively on their chemical

Table II Percent yield (w/w) of Propolis and Vespae Nidus extracts.

Sample	Water ext. (%)	EtOH-sol. (%)	EtOH-insol. (%)	MeOH ext. (%)	Ether sol. (%)	Ether-insol. (%)
P1	6.00	0.80	4.40	45.00	41.17	2.47
P2	6.88	0.65	4.38	39.00	36.10	2.80
P3	7.12	0.93	4.77	35.00	30.00	4.70
P4	7.48	0.71	5.16	42.50	38.96	3.48
P5	7.11	0.73	4.95	43.60	39.25	4.16
R1	24.16	0.008	2.00	4.46	1.00	1.00

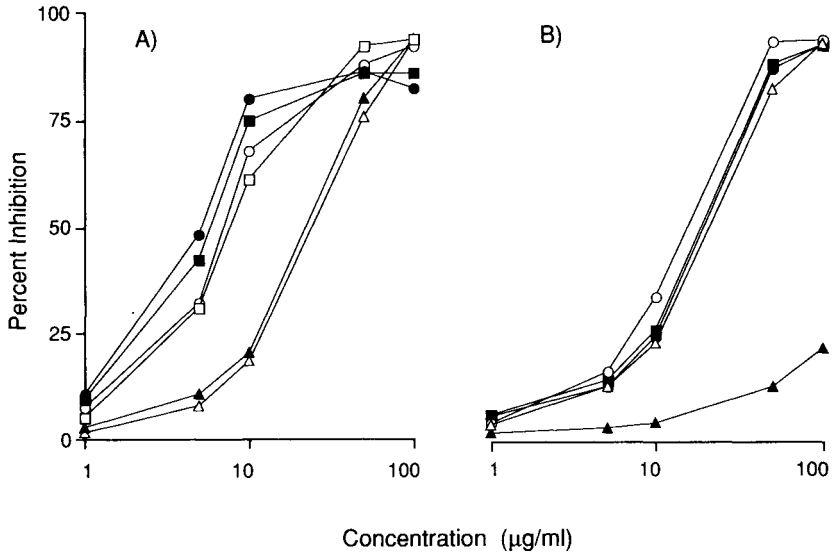

Fig. 1 DPPH radical scavenging effects of propolis and Vespae Nidus. The DPPH radical scavenging effect was measured by the absorbance of DPPH radical at 520 nm in a reaction containing the test sample and 30 mM DPPH. (A), Propolis ; (B), Vespae Nidus. Water extract (■), EtOH-soluble fr. (□), EtOH-insoluble fr. (●), MeOH extract (△), ether-soluble fr. (▲), ether-insoluble fr. (○).

constituents. The 2D-TLC on Avicel plates revealed that propolis differed each other in a few substances, and Vespae Nidus had a distinct pattern compared to them. The characterization of the chemical constituents through some spray reagents indicated several compounds visualized by 5 % alcoholic $FeCl_3$ solution, a spray reagent that gives dark green color for phenolic compounds, while Vespae Nidus presented the major substances being positive to 2 % ninhydrin solution, that detects amino acids and peptides.

Radical scavenging activities of the fractions of propolis from Lagoa Vermelha (P1) and Vespae Nidus from Hong Kong (R1)

For the comparative analysis of the radical scavenging activities of propolis (**P1**-**P5**) and Vespae Nidus (**R1**) initially **P1** was randomly selected for fractionation procedures and the same procedures were performed over **R1**. The values for 50 % radial scavenging activity (IC_{50}) are shown in Table III. As shown in Fig. 1, all extracts and fractions of **P1** and **R1**, except the ether-soluble fraction of **R1**, quenched the DPPH radical by 80 %-90 % at the concentrations

Table III Effects of propolis (**P1**-**P5**) and Vespae Nidus (**R1**) on scavening DPPH radical, and superoxide anion radical in xanthine/XOD and NAD/PMS reactions.

	Samples	IC_{50} (μg/ml)		
		DPPH	Xanthine/XOD	NADH/PMS
P1	Water ext.	5.8	3.6	34.0
	EtOH-sol.	8.0	8.0	110.0
	EtOH-insol.	5.2	3.2	14.0
	MeOH ext.	32.0	60.0	290.0
	ether-sol.	28.0	7.0	400.0
	ether-insol.	4.6	12.0	72.0
R1	Water ext.	18.0	17.0	88.0
	EtOH insol.	19.0	17.0	100.0
	MeOH ext.	21.0	20.0	200.0
	ether-sol.	>100.0	>100.0	>250.0
	ether insol.	16.0	5.8	18.0
P2	EtOH-insol.	5.4	4.4	12.0
	ether-insol.	4.2	3.6	56.0
P3	EtOH insol.	5.4	4.0	10.0
	ether insol.	8.0	6.8	65.0
P4	EtOH-insol.	5.6	2.8	14.0
	ether-insol.	6.0	7.0	50.0
P5	EtOH insol.	5.6	2.6	17.0
	ether-insol.	6.0	7.6	65.0
Caffeic acid		1.0	1.2	36.0
SOD			8.0×10^{3}	2.6

of 50-100 μg/ml. The IC_{50} values of **P1** for the DPPH radical show clearly that the water extract and its fractions, EtOH-soluble and -insoluble were potent scavengers, as well as the ether-insoluble fraction of the MeOH extract. The MeOH extract and its ether-soluble fraction presented values close to each other and were less active than the others. For **R1**, a similar pattern was observed but with low potency.

Similarly, the production of superoxide anion in the reaction of xanthine and XOD was inhibited by 72 %-100 % in the presence of the extracts and the fractions of **P1** and **R1**, except the ether-soluble fraction of **R1**, at the concentrations of 100 μg/ml (Fig. 2). The results given by this experiment were generally in good correlation with those of DPPH radical also on the IC_{50} values.

In the experiment for production of superoxide anion by a non-enzymatic reaction, by NADH/PMS, the water extract, the EtOH-insoluble and ether-insoluble fractions of **P1** showed 83 %, 82% and 62 % inhibition at 100 μg/ml, respectively (Fig. 3). It was necessary to increase the concentration of the extracts and fractions of **R1** to obtain the maximum

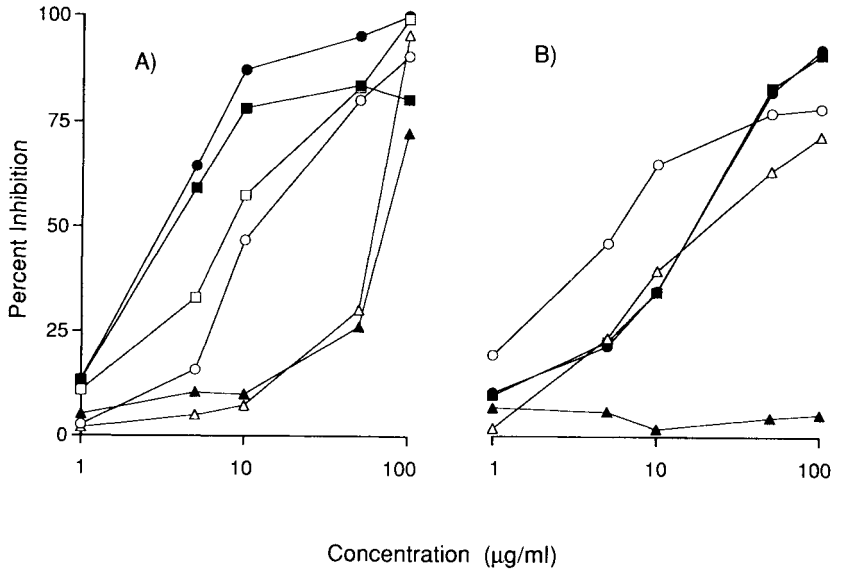

Fig. 2 Superoxide anion radical scavenging effects of propolis and Vespae Nidus. The superoxide anion radical scavenging effect was measured by the absorbance of NBT at 560 nm in a reaction containing 0.1 mM xanthine, 0.01 U/ml XOD, 0.1 mM EDTA, 0.05 mg/ml BSA, 0.025 mM NBT and 50 mM Na_2CO_3 buffer (pH 10.2). (A), Propolis ; (B), Vespae Nidus. Water extract (■), EtOH-soluble fr. (□), EtOH-insoluble fr. (●), MeOH extract (△), ether-soluble fr. (▲), ether-insoluble fr. (○).

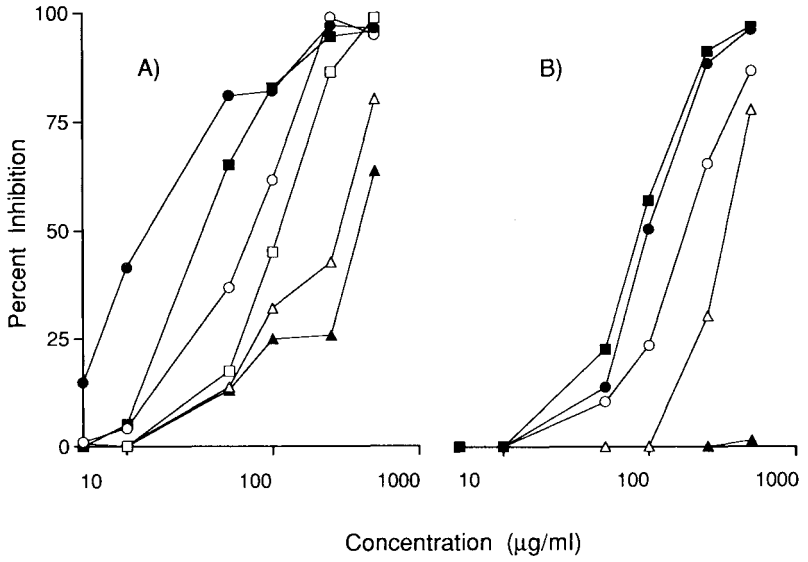

Fig. 3 Superoxide anion scavenging effects of propolis and Vespae Nidus. The superoxide anion radical scavenging effect was measured by the absorbance of reduced NBT at 560 nm in the presence of 0.156 mM NADH and 0.01 mM phenazyne methosulfate. (A), Propolis ; (B), Vespae Nidus. Water extract (■), EtOH soluble fr. (□), EtOH insoluble fr. (●), MeOH extract (△), ether soluble fr. (▲), ether-insoluble fr. (○).

inhibition of the superoxide anion production in this experiment. The IC_{50} also showed some correlation with the above tests however the values were higher, especially for the MeOH extract and its ether-soluble fraction.

From the above experiments it was found that the scavenging effects of DPPH and superoxide anion radicals were present in both, **P1** and **R1**, at different potencies. The water extracts and its EtOH insoluble fraction, and the ether-insoluble fraction of MeOH extract showed the most potent effects. The order of the potency was EtOH-insoluble fraction=ether-insoluble fraction>water extract>EtOH-soluble fraction>MeOH extract>ether-soluble fraction. Generally the effects of **P1** were stronger than those of **R1**.

Comparative tests for the radical scavenging activity in propolis from different regions in Brazil (P1, P2, P3, P4 and P5)

From the previous study on the fractions of **P1** for radical scavenging activity the EtOH-insoluble and ether insoluble fractions were found to possess the strongest activities. Therefore the same fractions of propolis collected in different regions in Brazil (**P1**, **P2**, **P3**, **P4** and **P5**) were compared by the experimental methods described above. Their IC_{50} were similar to each other in all 3 experiments. For DPPH radical scavenging activity the IC_{50} of **P2**-**P3** were 5.4-5.6 μg/ml for the EtOH-insoluble fractions and 4.2-8.0 μg/ml for the ether-insoluble fractions. For the xantine/XOD reaction the IC_{50} were 2.6-4.4 μg/ml for the EtOH-insoluble fractions and 6.8-7.0 μg/ml for the ether insoluble fractions. In the reaction of NADH/PMS, the inhibitory effects shown were slightly lower, the IC_{50} being 10.0-17.0 μg/ml for the EtOH insoluble fractions and 50.0-65.0 μg/ml for the ether-insoluble fractions. Fig. 4 shows the DPPH and superoxide anion radical scavenging effects by the EtOH-insoluble fractions of **P1**, **P2**, **P3**, **P4** and **P5**. No significant differences on the radical scavenging activities were observed in different samples of Brazilian propolis.

Effect of the EtOH-insoluble fractions of propolis (P1-P5) and R1 on XOD

An additional experiment on XOD was carried out. The EtOH-insoluble fraction of each propolis and **R1** were tested for the inhibitory effects of XOD. As

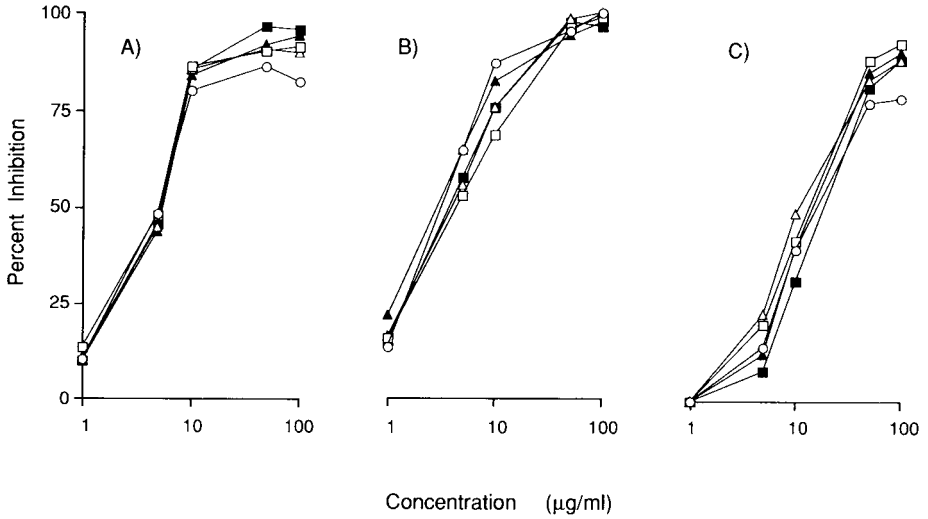

Fig. 4 Comparative superoxide anion and DPPH radicals scavenging effects of propolis from various sources.(A), DPPH ; (B), superoxide anion (xanthine-XOD system) ; (C), superoxide anion (PMS-NADH system). The samples tested were the EtOH-insoluble fractions of the water extracts of P1 (○), P2 (□), P3 (△), P4 (▲) and P5 (■).

a result, the inhibitory effect was not observed in any of the samples tested.

Discussion

The free radical scavenging activities of propolis were reported earlier by Scheller[3] in an experiment using propolis from Poland, and recently by Pascual[4] on Cuban propolis. In the present study a qualitative chemical analysis was performed on propolis and Vespae Nidus that showed no notorius differences between propolis of different regions but distinct differences between propolis and Vespae Nidus on the yield of the extraction and on the main chemical constituents. On the test for their biological activities firstly the DPPH and superoxide anion radical scavenging activities were monitored in the presence of the extracts of a Brazilian propolis (**P1**) and Vespae Nidus (**R1**). Although in general **P1** showed to be stronger than **R1** in the present radical scavenging tests there was no significant difference between propolis of different sources (**P1-P5**). Neither propolis nor **R1** influenced the activity of XOD and this suggests that the effects of the extracts and the fractions of propolis (**P1-P5**) and **R1** were mainly due to the scavenging of oxygen radicals formed in the reaction and not due to the inhibition of XOD activity.

SOD is an enzyme that catalyzes the dismutation of univalently reduced oxygen formed in many biological oxidations and is assumed to play an important role in aerobic organisms for defense against the deleterious actions of the superoxide radical.[9] Therefore SOD-like activity is expected to be effective against affections involving active oxygen free radicals, such as inflammation, tumor, atherosclerosis, melanin pigmentation, *etc.* In the present study caffeic acid was used as a positive control since it is a substance commonly present in plants and is known to have radical scavenging activity. In addition, SOD was tested in the reactions of xanthine/XOD and NADH/PMS and the strong inhibition shown by this ensured the involvement of superoxide in both reactions. The free radical scavenging effects of the propolis and **R1** extracts were not as strong as those of caffeic acid or SOD, however, the mild effects should not be unconsidered before a more detailed study *in vivo*.

A number of experimental pharmacological

effects of propolis have been reported.[10] On the other hand, there is a report on 100 cases of clinical application of a Chinese medicine prescription containing Vespae Nidus (複方露蜂房滴鼻液) for the treatment of rhinitis.[11] While there are only a few studies on the chemical constituents of Vespae Nidus, several constituents of propolis were identified so far, most of them flavonoids and caffeic acid derivatives. Flavonoids have been identified in propolis of Brazil,[12] Bulgaria[13] and Spain,[14] *etc.*, and other propolis of different origins have been studied quantitatively and qualitatively.[15] Some reports suggest the quantitative determination of flavonoid compounds for the standardization of propolis.[11,16] However, additional tests are required for the evaluation of the products, since propolis is a complex of many substances coming from the exudates of plants and the bee itself, in which the content of chemical constituents (including flavonoids) vary qualitatively and quantitatively with the environment. The identification of Vespae Nidus is relatively simple by the morphology while satisfactory evaluation of propolis is very difficult since it is commercialized in several forms, from the crude solid forms to extracts or solutions, and also in different range of concentrations. Moreover, the commercial propolis probably are mixture of raw materials collected in different apiaries. Therefore the present results showing that there are no remarkable differences between propolis of different regions in Brazil indicate that there should not be a great influence by those materials on the biological activity concerning radical scavenging effects. However, the standardization of propolis on its biological activities and on the chemical constituents is very important. This kind of comparative study is an initial work that is going to be performed on propolis from different regions in the world. Furthermore this also served as a trigger for a re-evaluation of the use of Vespae Nidus and its medicinal properties.

Acknowledgments

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和文抄録

プロポリスはミツバチが樹木の蕾や樹皮から採集した物質と自らの分泌物を混合したものであり，古くから民間薬として用いられている。一方，露蜂房はスズメバチ科の蜂の巣を乾燥した漢薬である。これらは共にハチの採集生産物である他，薬理作用や適応領域にも共通点が見いだされている。プロポリスはフリーラジカル消去作用を有することが報告されており，この作用がプロポリスの薬効に関与していることが考えられる。今回，活性酸素消去作用に関するプロポリスと露蜂房の比較検討を行い，両者にその作用が存在することを見いだした。またその作用は水溶性の画分に最も強く認められた。

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